Changes in Essential Oil during Enzyme-Assisted Ensiling of Lemongrass (*Cymbopogon citratus* Stapf.) and Lemon Eucalyptus (*Eucalyptus citriodora* Hook)

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Changes in essential oil during ensiling of lemongrass and lemon eucalyptus were studied. Wilted lemongrass and eucalyptus leaves were ensiled in 0.25-L anaerobic jars. Samples consisted of a control (no additives) and a treated sample (0.5% glucose and lactic acid bacteria and 1% cellulase plus 1% hemicellulase plus pectinase). Three jars per treatment were sampled on days 2, 6, 10, and 36 for analysis of essential oil. Essential oil was obtained by extraction and by hydrodistillation. Extraction efficacy of essential oil from the lemongrass was improved by the enzyme treatment, but it was much lower than the amount obtained by distillation. The major components of the essential oil were neral and geranial. In the eucalyptus, total essential oils obtained by distillation decreased during ensiling, and the amount was similar to the amount obtained by extraction. Citronellal, which was the major component of the essential oil in the fresh eucalyptus leaves, decreased, whereas isopulegol and 3,8-terpinolhydrate increased during ensiling.

Keywords: *Essential oil; lemongrass; lemon eucalyptus; ensiling; enzyme*

INTRODUCTION

Plants contain a large variety of valuable natural products including nutrients, drugs, fragrances, colorants, antioxidants, etc. In many cases their extraction involves drying, heat, and use of various solvents. Many of the valuable natural materials are present in the plants in small quantities, and are sensitive to the sometimes harsh extraction conditions.

An alternative process, which might improve the recovery of natural products from plants, is enzyme-assisted ensiling (ENLAC). Ensiling, a preservation method for moist crops, is based on natural solid-state fermentation whereby lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid. As a result the pH decreases and the forage is preserved. Cell-wall hydrolyzing enzymes (cellulases, hemicellulases, and pectinases) can be applied in the ensiling of sugar-poor forage crops, and the sugars released from partial hydrolysis of the cell wall can be used by the LAB in the ensiling fermentation (e.g. *1*, *2*). Enzyme levels commonly used in forage ensiling are 0.025−0.05% on a fresh weight basis.

The ENLAC process can also be used to improve the extractive recovery of valuable substances from plants. Following are the advantages of ENLAC in comparison with conventional methods: (1) Partial hydrolysis of the cell wall by ENLAC increases its permeability and facilitates higher recovery yields of the target materials. (2) Preservation of the plants by ensiling makes long-term storage possible, eliminating the need for immediate processing. (3) Softening of the cell wall by ENLAC ensures mild extraction conditions. (4) The anaerobic conditions of the process result in negative redox potentials and protect susceptible materials against oxidation. (5) The pH values obtained by ENLAC (4.5−5.0) are compatible with the optimal pH requirements of the cell wall hydrolyzing enzymes.

Previous studies have proven the efficacy of ENLAC in the recovery of protein, chlorophyll, β-carotene, and xanthophyll from alfalfa; preservation of sugar in sweet sorghum; and recovery of polyphenols from sage and rosemary (*3*−*6*).

The odors of many plants such as roses, lavender, citronella, eucalyptus, peppermint, etc., are due to the presence of volatile C_{10} and C_{15} compounds which are called terpenes. These compounds, referred to as essential oils, can be recovered by hydrodistillation or ether extraction. Of particular importance are cyclic series of the alcohols geraniol, nerol, and linalool, which occur in rose and other flower essences. Aldehydes such as geranial, neral, and citronellal, have citrus-like odors (*7*).

Lemongrass (*Cymbopogon citratus* Stapf.) and lemon eucalyptus (*Eucalyptus citriodora* Hook) contain citronellal, neral, and geranial, which are used as flavor and fragrance ingredients. The purpose of the present study was to examine the changes in essential oil composition during the enzyme-assisted ensiling of lemongrass and lemon eucalyptus.

MATERIALS AND METHODS

Materials. Lemongrass was harvested and allowed to wilt in the field for 3 h. Eucalyptus leaves were separated from the branches manually. The leaves were chopped into 3−5-

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Table 1. Chemical Analysis of the Lemongrass and Eucalyptus: Fresh Materials and Silages after 36 Days[a]

treatment	DM	pH	WSC[b]	lactic acid[b]	ethanol[b]	acetic acid[b]	NDF[b]	ADF[b]	ADL[b]
				Lemongrass					
fresh material	333	5.7[a]	40[a]				563[a]	283[b]	29[a]
control silage	306	5.4[b]	14[b]	5[b]	9	2	576[a]	303[a]	27[a]
treated silage	310	4.1[c]	46[a]	31[a]	7	4	470[b]	245[c]	20[b]
				Eucalyptus					
fresh material	394	5.3[a]	15[b]				289[b]	231[b]	100[b]
control silage	379	4.5[b]	12[b]	NF	2	NF	337[a]	306[a]	158[a]
treated silage	391	4.0[a]	64[a]	traces	1	1	305[a,b]	273[a,b]	137[a,b]

[a] DM, dry matter, in grams per kilogram; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin. Within a column and plant, means followed by different letters differ significantly ($P < 0.05$). [b] In grams per kilogram of dry matter.

Table 2. Changes in Composition of MTBE-Extracted Essential Oils during Ensiling of Lemongrass Leaves (Milligrams per Gram)

	time of ensiling (days)								
	0	2		6		10		36	
compound[a]	control	control	treated	control	treated	control	treated	control	treated
myrcene	0.012 ± 0	0.017 ± 0.003	0.013 ± 0.005	0.026 ± 0.013	0.019 ± 0.002	0.012 ± 0.003	0.016 ± 0.001	0.026 ± 0.003	0.053 ± 0.011
nerol	0	0.014 ± 0.004	0	0	0.002 ± 0	0.007 ± 0.007	0.002 ± 0	0.002 ± 0.001	0.003 ± 0.003
neral	0.053 ± 0.04	0.032 ± 0.008	0.040 ± 0.010	0.105 ± 0.064	0.072 ± 0.012	0.080 ± 0.015	0.078 ± 0.011	0.115 ± 0.088	0.318 ± 0.073
geraniol	0.015 ± 0.002	0.057 ± 0.025	0.012 ± 0.005	0.029 ± 0.014	0.017 ± 0.006	0.021 ± 0.006	0.018 ± 0.003	0.025 ± 0.016	0.051 ± 0.010
geranial	0.112 ± 0.004	0.062 ± 0.014	0.094 ± 0.025	0.190 ± 0.107	0.151 ± 0.021	0.139 ± 0.026	0.150 ± 0.024	0.170 ± 0.130	0.524 ± 0.121
geranic acid	0.004 ± 0.0001	0.003 ± 0.003	0.002 ± 0.001	0.011 ± 0.003	0.005 ± 0.002	0.002 ± 0.002	0.005 ± 0.001	0.016 ± 0.003	0.016 ± 0.003
geranyl acetate	0.006 ± 0	0.003 ± 0.003	0.004 ± 0.002	0.010 ± 0.007	0.006 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.014 ± 0.002	0.019 ± 0.006
total extract	0.203 ± 0.009	0.192 ± 0.057	0.166 ± 0.046	0.380 ± 0.210	0.296 ± 0.045	0.269 ± 0.052	0.312 ± 0.039	0.375 ± 0.234	1.013 ± 0.211
silage pH	5.7	5.8	5.0	5.9	4.3	5.9	4.1	5.3	4.1

[a] In addition, some samples also contained traces of 6-methyl-5-heptene-2-one.

cm pieces and ensiled in 250-mL anaerobic jars, either with no further treatment (control) or for treatment with glucose−LAB inoculant-enzymes. Each jar was filled with ~80 g (wet weight) of plants, without a headspace. The jars were stored at room temperature (25−27 °C). Three jars per treatment were sampled on days 2, 6, 10, and 36 for essential oil analysis. Three additional jars per treatment were sampled on day 36 for analysis of fermentation end products and cell-wall composition.

The treatment included 0.5% glucose, H/M F Medipharm USA inoculant (Medipharm USA, Des Moines, IA), and 1% cellulase and 1% (hemicellulase plus pectinase) (w/wet weight). The inoculant contained 5×10^9 colony forming units (cfu) per gram of *Lactobacillus plantarum*, *Enterococcus faecium*, and *Pediococcus acidolactici* (manufacturer's declaration). It was applied by suspending 200 mg of the inoculant powder in 10 mL of water, spraying it over 1.5 kg of plants, and mixing thoroughly. Thus, about 7×10^5 cfu/g were applied.

The enzymes used were Celluclast 1.5L and Peelzyme II (Novo Nordisk, Switzerland). The stated activities of the enzymes were 1500 NCU (Novo cellulase units) and 3500 FDU 55°C (Ferment Depectinisation Units) mL^{-1}, respectively. Thus, 10 NCU and 2 FDU 55°C were added per gram of silage.

Analytical Procedure. Dry matter was determined by oven drying for 48 h at 60 °C. Water-soluble carbohydrates (WSC) were determined according to the phenol−sulfuric acid method (*8*). Lactic acid was determined by a colorimetric method (*9*). Volatile fermentation end products (ethanol, and acetic, propionic, and butyric acids) were determined by gas chromatography with an FFAP (Hewlett-Packard) megabore column. Helium was used as a carrier gas. Injection and detector temperature was 240 °C. Column conditions were 45 °C for 1 min, followed by 35 °C/min to 120 °C, 1 min at 120 °C, followed by 30 °C/min to 230 °C. The fermentation products were identified with an FID detector using valeric acid as an internal standard.

Fiber analysis included the determination of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL); fiber analysis was performed according to ref *10*, with an Ankom filter bag system (Ankom Technology Corp., Fairport, NY).

Essential oils were extracted according to refs *11* and *12*: A plant sample (about 1 g fresh weight) was extracted with 10 mL of methyl-*tert*-butyl-ether (MTBE) containing 10 mg/mL isobutylbenzene as an internal standard, for 24 h, with gentle shaking at room temperature. The extract was passed through a small column (comprising a Pasteur pipet) containing anhydrous Na_2SO_4 and salicylic acid (Silicagel 60, 230−400 mesh, Merck) to dry it and to remove high-molecular-weight polar substances that would interfere with the GC analysis. In addition to the extraction, samples of at least 250 g of fresh plant material were hydro-distilled for 1.5 h in a Clevenger type apparatus, with 700 mL of water. The essential oil was cooled and separated from the cohobated water (*13*), and samples comprising 1.0 μL of distilled essential oils (diluted 1:10 000 in hexane) or undiluted MTBE extracts were analyzed on a HP-GC model D apparatus equipped with an HP5 (30 m × 0.25 mm) fused-silica capillary column. Helium was used as a carrier gas. Injection temperature was 250 °C, and detector temperature was 280 °C. Column conditions were 70 °C for 2 min, followed by 4 °C/min to 200 °C. Identification of the main components was done by co-injection of authentic standards and comparison of the EIMS (electron impact mass spectroscopy) obtained with that of computerized libraries (*12*).

Statistical analyses of silage parameters included one-way analysis of variance (ANOVA) and Duncan's multiple range test, performed with the GLM (general linear model) procedure of the Statistical Analysis System (SAS, Cary, NC).

RESULTS

Table 1 gives the chemical analyses of the fresh lemongrass and eucalyptus and of their respective silages. The enzyme treatment resulted in lower pH values throughout the ensiling period (Tables 2 and 4); it also gave the higher WSC contents, because of the sugar released by cell-wall hydrolysis. In the lemongrass control silages, ethanol and lactic acid were the major fermentation products, whereas much higher levels of lactic acid were found in the treated silages, which is consistent with the lower pH values. In the eucalyptus

Table 3. Changes in Composition of Hydrodistilled Essential Oils during Ensiling of Lemongrass Leaves (Milligrams per Gram)

	time of ensiling (days)								
	0	2		6		10		36	
compound[a]	control	control	treated	control	treated	control	treated	control	treated
myrcene	0.42	0.50	0.30	0.23	0.18	0.12	0.20	0.45	0.21
nerol	0.04	0.03	0.03	0.03	0.03	0.04	0.02	0.13	0.10
neral	3.42	3.12	3.04	3.39	3.15	3.13	3.03	3.19	2.70
geraniol	0.59	0.52	0.54	0.64	0.59	0.57	0.56	0.62	0.50
geranial	4.8	4.29	4.43	4.93	4.41	4.49	4.48	4.44	3.92
geranic acid	0.02	0.01	0.04	0.04	0.03	0.02	0.04	0.05	0.03
geranyl acetate	0.35	0.38	0.43	0.42	0.40	0.36	0.36	0.38	0.34
total essential oil	10.00	10.78	9.30	9.68	9.05	9.09	9.10	9.35	8.44

[a] In addition, some samples also contained traces of 6-methyl-5-heptene-2-one, linalool, mentha-1,5-diene-8-ol, and unidentified compounds.

Table 4. Changes in Composition of MTBE-Extracted Essential Oils during Ensiling of Lemon Eucalyptus Leaves (Milligrams per Gram)

	time of ensiling (days)								
	0	2		6		10		36	
compound[a]	control	control	treated	control	treated	control	treated	control	treated
isopulegol	0.01 ± 0.01	0.52 ± 0.18	0.57 ± 0.03	0.68 ± 0.08	0.96 ± 0.25	0.76 ± 0.13	1.26 ± 0.32	1.63 ± 0.27	2.02 ± 0.51
citronellal	8.33 ± 0	6.52 ± 0.16	8.89 ± 0.96	4.37 ± 0.73	7.14 ± 0.20	3.37 ± 0.45	4.86 ± 1.47	3.57 ± 0.49	2.52 ± 0.76
citronellol	0.27 ± 0.01	0.60 ± 0.06	0.49 ± 0.07	0.43 ± 0.04	0.51 ± 0.04	0.58 ± 0.06	0.45 ± 0.09	0.46 ± 0.06	0.51 ± 0.11
citronellic acid	0	0.07 ± 0.02	0.07 ± 0.01	0.10 ± 0.06	0.08 ± 0.03	0.07 ± 0.01	0.045 ± 0.01	0.09 ± 0.02	0.09 ± 0.03
3,8-terpineol-hydrate	0	0.98 ± 0.61	1.07 ± 0.15	1.44 ± 0.34	1.93 ± 0.34	1.41 ± 0.29	2.69 ± 0.67	3.54 ± 0.71	5.00 ± 0.82
total extract	9.1 ± 0.3	9.8 ± 1.1	12.0 ± 1.3	8.6 ± 0.8	12.3 ± 0.5	7.3 ± 0.8	11.2 ± 2.7	11.9 ± 2.1	13.7 ± 1.5
silage pH	5.3	5.0	4.6	4.8	4.2	4.9	4.2	4.8	4.2

[a] In addition, some samples also contained traces of β-pinene, 1,8-cineol, and unidentified compounds.

silages, almost no fermentation products were found, and it might well be that they were produced but underwent some reaction with plant metabolites or were transformed by microorganisms. In the lemongrass, the enzyme treatment resulted in much lower cell-wall (NDF and ADF) contents than in the control silages or in the fresh plants. In the eucalyptus, the silages had higher NDF, ADF, and ADL contents than the fresh plants, and there was less effect of the enzymes on cell-wall content.

Table 2 gives the changes in the essential oils of the lemongrass in the MTBE extracts. Recovery of essential oils from the control silages did not increase significantly relative to that from fresh plants, whereas the recovery from the enzyme-treated silages increased markedly after 36 days of ensiling. This was because in lemongrass the essential oil is confined within specific lignified cells in parenchymal tissues in the leaves (*11*) and may be released when the leaf tissues are softened by the enzymes. In the distillation, the efficacy of oil recovery is maximal, and the essential oil recovered by distillation represents the total content. The results indicate that there was a 10−20% decrease in the total essential oils which were recovered after ensiling, with no marked differences between the control and treated silages. (Table 3). It could be that some of the essential oil was consumed by microorganisms or destroyed under the acidic condition of the silage. Recovery of essential oil by extraction, even from enzyme-treated silages, was much lower (on the order of micrograms per gram) than that by distillation (of the order of miligrams per gram).

In the initial stages of fermentation, the essential oil which was free and available was obtained in the extract, and it was affected by the ensiling process (Table 2). In later stages, as the silage stabilized and the plants softened, more essential oil was recovered which had not been affected by the fermentation. On day 2, nerol and geraniol increased concomitantly with decreases in geranial and neral. The appearance of the alcohols nerol and geraniol in the extract was related to reduction of the aldehydes neral and geranial under the anaerobic conditions of the silage. From day 6 onward, the geranial and neral contents increased, more so in the extract from enzyme-treated silages, and they were the major components of the extract. Increases in geranic acid and geranyl acetate were related to the enhanced extraction efficiency of these compounds in the later stages of ensiling, because of tissue softening. In the distillate (Table 3) the composition of the essential oil remained generally constant throughout ensiling, and the relative decreases of various components reflected the general decrease in total essential oil.

Table 4 gives the changes in the essential oils of the eucalyptus in the MTBE extracts. The essential oils content of the fresh leaves comprised only 50% of the total essential oil, the latter being represented by the oil recovered by distillation (Table 5). The total extracted increased as ensiling proceeded, more so in the enzyme-treated silages, and after 36 days it was similar to the amount obtained by distillation. This result was different from that obtained with lemongrass, where the extract comprised only a small fraction of total essential oil obtained by distillation. The difference between the two plants is explained by difference in the manners in which the essential oil accumulates in the tissues. Whereas in lemongrass the oil is located in specific lignified cells in parenchymal tissues in the leaves (*14*), in eucalyptus it is stored in secretory cavities located in the epidermal tissue (*15*). Total essential oil recovered by distillation decreased by 33% during ensiling, and we suggest that the composition of the essential oil in

Table 5. Changes in Contents of the Hydrodistilled Major Essential Oil Components during Ensiling of Lemon Eucalyptus Leaves (Milligrams per Gram)

	time of ensiling (days)								
	0	2		6		10		36	
compound[a]	control	control	treated	control	treated	control	treated	control	treated
isopulegol	1.0	2.2	2.3	1.8	2.7	2.5	1.3	3.4	5.3
citronellal	17.5	14.7	12.2	9.7	8.7	10.9	8.4	6.2	4.4
citronellol	1.0	2.1	1.3	1.5	1.3	1.9	1.3	1.8	1.2
citronellic acid	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
3,8-terpineolhydrate	0.0	0.4	1.0	0.9	1.4	0.8	1.2	1.2	1.5
citronellyl acetate	0.3	0.2	0.5	0.4	0.4	0.4	0.5	0.6	0.4
total essential oil	20.5	20.0	17.8	14.6	15.4	16.7	14.6	13.7	14.1

[a] In addition, some samples also contained traces of β-pinene, 1,8-cineol, α-terpinolene, and unidentified compounds.

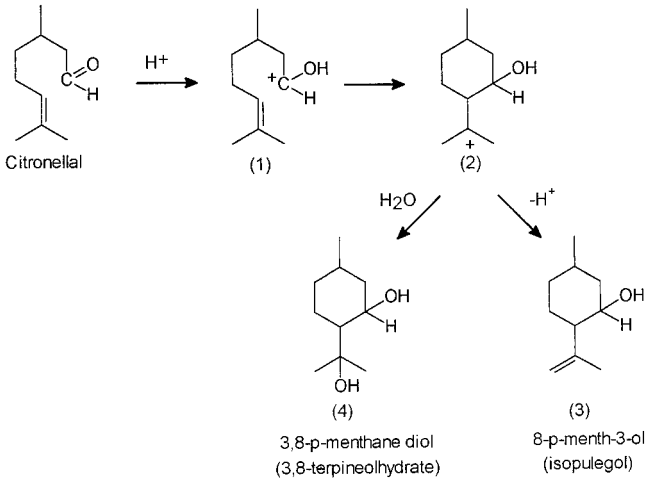

Figure 1. Acid-catalyzed Prins-type condensation of citronellal. The mechanism is an electrophilic attack on the C═C and C═O double bonds. The acid first protonates the C═O, and the resulting carbonium ion (1) attacks the C═C. The resulting (2) may undergo loss of H^+ to give the olefin (3), or may add water to give the diol (4).

both the extract and the distillate might have changed considerably during ensiling, and that compounds which could not be detected by our analytical method were produced under the acid and reducing conditions which prevailed in the silage. Therefore, a decrease in total essential oil was observed. The most conspicuous changes were the appearance of isopulegol and 3,8-terpineolhydrate, which were not present on day 0. There was also some increase in citronellol, caused by reduction of citronellal and products obtained by Prins-type condensation (*16*). It is interesting to notice that in the extract 3,8-terpineolhydrate was the major new component, whereas in the distillate, isopulegol was present at higher levels than the former. The mildly acidic conditions of the ensiling process provide a self-condensation between the double bond and the aldehyde groups of citronellal (an open chain monoterpene) to produce isopulegol and 3,8-terpineolhydrate (cyclic monoterpenes) (Figure 1).

DISCUSSION

The current experiments were performed in the hope that the ENLAC process would increase the efficiency of extraction of essential oil from lemongrass and lemon eucalyptus. In previous studies ENLAC resulted in increased extraction efficiency of various components, such as leaf protein and chlorophyll from alfalfa and total polyphenols from sage and rosemary (*5*, *6*). In the current study ENLAC also increased the recovery of essential oil by extraction as compared with control silages, and this is attributed to partial cell-wall hydrolysis by the cell-wall hydrolyzing enzymes.

However, recovery of essential oils from lemongrass by extraction was much lower than their recovery by distillation, the latter representing the total essential oil which is available. This was true also when recoveries of essential oil from fresh eucalyptus leaves by extraction and distillation were compared. However, recoveries of essential oil from eucalyptus by extraction and distillation after ensiling were similar. The difference between lemongrass and eucalyptus is attributed to the differing availabilities or manners of storage of the essential oil in the two plants. It is interesting to notice that in the lemongrass silages the enzyme treatment reduced cell-wall content considerably, whereas in the eucalyptus their effect was smaller (Table 1); this was reflected in the higher efficiency of extraction from the enzyme-treated lemongrass silages as compared with that from the control silages (Table 2), whereas recoveries of essential oil by extraction from eucalyptus silages were similar in the control and enzyme-treated cases (Table 4).

The results reveal that total essential oil obtained by distillation decreased during 36 days of ensiling in both plants. It seems that this fraction is sensitive to the acidic conditions prevailing in the silage. In addition to the decrease in quantity, ensiling also resulted in changes in the composition of the essential oils, and the final product obtained after ensiling was different from the one which was recovered from the fresh plants. This has extensive implications for odor quality. For example, in the eucalyptus, citronellal (citrus-like odor) was self-condensed to isopulegol and 3,8-terpineolhydrate, which have different odor profiles. In the lemongrass, however, citral was reduced to nerol and geraniol. These different odor notes could be identified by laboratory personnel. Such changes can be brought about by plant enzymes (*12*) or by various microorganisms (*17*).

In developing ENLAC technology for extraction of valuable materials from plants, it is important to study not only extraction efficiency, but also changes that might be caused in the target substances by the ensiling process. In the case of lemongrass and eucalyptus, ENLAC resulted in both a decrease in total essential oil and changes in its composition which were not always desirable from the aspect of its odor profile. It would be worthwhile, however, to test ENLAC further in different applications.

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